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Role of *SDHAF2* and *SDHD* in von Hippel-Lindau associated pheochromocytomas

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Abstract

Pheochromocytomas (PCCs) develop from the adrenal medulla and are often part of a hereditary syndrome such as von Hippel Lindau (VHL) syndrome. In VHL, only about 30% of patients with a *VHL* missense mutation develop PCCs. Thus, additional genetic events leading to formation of such tumors in patients with VHL syndrome are sought for. *SDHAF2* (previously termed *SDH5*) and *SDHD* are both located on chromosome 11q and required for the function of mitochondrial complex II. While *SDHAF2* has been shown to be mutated in patients with paragangliomas (PGLs), *SDHD* mutations have been found in both patients with PCCs and PGLs. Since loss of 11q is a common event in VHL-associated PCCs, we aimed to investigate whether *SDHAF2* and *SDHD* are targets. In this study, 41 VHL-associated PCCs were screened for mutations and loss of heterozygosity (LOH) in *SDHAF2* or *SDHD*. Promoter methylation as well as mRNA expression of *SDHAF2* and *SDHD* was studied. In addition, immunohistochemistry (IHC) of *SDHB*, known to be a universal marker for loss of any part the SDH complex, was conducted. LOH was found in more than 50% of the VHL-associated PCCs, and was correlated to a significant decrease ($p<0.05$) in both *SDHAF2* and *SDHD* mRNA expression, which may be suggestive of a pathogenic role. However, while *SDHB* protein expression as determined by IHC in a small cohort of tumors was lower in PCCs than in the surrounding adrenal cortex, there was no obvious correlation with LOH or the level of *SDHAF2/SDHD* mRNA expression. In addition, the lack of mutations and promoter methylation in the investigated samples indicate that other events on chromosome 11 might be involved in the development of PCCs in association with VHL syndrome.

Introduction

Pheochromocytomas (PCCs), also termed adrenal paragangliomas (PGLs), are tumors derived from chromaffin cells in the adrenal medulla while PGLs develop from neural crest-derived paraganglia of the autonomic nervous system and can be found throughout the body from the pelvis to the head and neck [1]. They frequently cause hypertension due to excessive catecholamine production and secretion. With the current data related to discoveries of new genes in the pathogenesis of PCCs and PGLs, the proportion of hereditary tumors is estimated to be around 30-35% [2-4]. The vast majority of these patients belong to one of the following syndromes: von Hippel-Lindau (VHL) [5], multiple endocrine neoplasia type 2 (MEN 2) [6], von Recklinghausen (NF1) [7] or pheochromocytoma-paraganglioma syndrome (PGL types 1-4) [8-11], which are caused by germline mutations in *VHL*, *RET*, *NF1* and *SDHx* (*SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2/SDH5*), respectively. In addition, germline mutations in *KIF1B β* have been found in patients with PCCs, neuroblastomas and other neural and non-neural tumors [12, 13]. More recently, *EPAS1/HIF2A* germline mutations were found in patients with PCCs/PGLs and polycythemia (polycythemia-paraganglioma syndrome) [14]. Susceptibility genes for which no syndromic form has been described yet include *EGLN1* [15], *TMEM127* [16] and *MAX* [17].

The majority of patients with VHL-associated PCCs have missense *VHL* germline mutations [18]. While probably less than 1% of patients with nonsense, microdeletion/insertion, or deletion germline mutations of *VHL* develop PCCs, about 30% of patients with missense germline mutations do so. Since only a subset of patients with missense *VHL* germline mutations develop PCCs, additional genetic events contributing to the formation of such tumors are sought after. Loss involving the long arm of chromosome 11 (11q) has been reported in almost 70% of VHL-associated PCCs [19] but no specific additional PCC triggering gene has yet been identified.

The SDH complex (mitochondrial complex II) plays an important role in the electron transport chain and the Krebs cycle [20]. *SDHAF2* (succinate dehydrogenase complex assembly factor 2), a gene located on chromosome 11q12.2, encodes a protein that interacts with the catalytic subunit of the SDH complex and is required for SDH-dependent respiration. *SDHAF2* was shown to act as a tumor

suppressor [21], and mutations in the gene were detected in unrelated families with hereditary PGLs [21, 22]. *SDHD* (succinate dehydrogenase complex, subunit D, integral membrane protein) is located on chromosomal region 11q23. The gene encodes a protein forming one of two transmembrane subunits in the SDH complex, important for the function of the electron transport chain [20].

This study investigates whether *SDHAF2* and/or *SDHD* are targets of additional altering events within 11q that could promote PCC development in carriers of *VHL* germline mutation. A panel of *VHL*-associated PCCs was screened for mutations and loss of heterozygosity (LOH) in *SDHAF2* and *SDHD*. Promoter methylation was investigated as an alternative mechanism of gene inactivation, and levels of mRNA expression of *SDHAF2* and *SDHD* were analyzed in tumors with and without LOH. Finally, protein expression studies were conducted by staining for the SDHB protein since it is considered a universal marker for loss of any of the SDH subunits [23].

Materials and Methods

Patients and samples

Forty-one PCCs from 40 patients with a *VHL* missense germline mutation were studied. For 19 of these tumors paired blood samples were available for LOH analysis. The samples were obtained through the University of Freiburg, Germany, the University of Padova, Italy, and the University of Texas Health Science Center, USA. Tumor and blood samples were snap frozen in liquid nitrogen and stored at $\leq -70^{\circ}\text{C}$, or in some cases stored in RNeasy lysis buffer (Qiagen, Crawley, UK). DNA was isolated using TRIzol (Invitrogen, Paisley, UK) or previously published methods [19], and RNA was isolated using TRIzol (Invitrogen). Informed consent was given in all cases and the local ethic committees approved the study.

LOH analysis by microsatellite genotyping

Tumor and blood samples were genotyped for microsatellite markers D11S4191, D11S1765, D11S4076 and D11S1883 which are all in close proximity to *SDHAF2*, as well as D11S1793, D11S1986, D11S897, D11S3179 in close proximity to *SDHD* (Table 1).

For each marker, 50 ng of DNA was amplified for 35 cycles using HotStar Taq Polymerase (Qiagen, Hilden, Germany) with primers and annealing temperatures as specified in Table 1. Forward primers were labeled with 6-FAM fluorophore (Sigma-Aldrich, St. Louis, MO). Amplicons were denatured at 95 °C and separated by capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). 600-LIZ (Applied Biosystems) was used as a size standard and results were analyzed with GeneMapper v. 4.0 (Applied Biosystems). Tumors were regarded as positive for LOH when the mean allele ratio between tumor and blood, calculated as earlier described [24], was <0.7 for all informative markers and apparent differences between tumor and blood allele ratios were seen in the electropherograms. In cases where no blood was available, allele peak ratios were compared to blood samples with the same or very similar allele combinations, followed by careful visual inspection of the electropherograms. Obvious allelic imbalance, i.e. large differences in allele peak heights and/or a higher peak in the longest allele were regarded as indicative of loss. Cases inconclusive at visual inspection and/or with one or more allele ratios >0.7 but <0.8 were regarded as inconclusive for LOH.

DNA sequencing

SDHAF2 and *SDHD* both contain 4 exons and 3 introns. Primers were designed to amplify exons 1-4, for each gene, including the 5'- and 3'-untranslated regions (Table 2). For each exon, 50 ng of tumor DNA was PCR-amplified for 35 cycles using Thermo White Taq DNA Polymerase (Saveen Werner, Limhamn, Sweden) at annealing temperatures as specified in Table 2. PCR products were purified from unconsumed primers and dNTPs with ExoSAP-IT (GE Healthcare, Waukesha, WI) and Sanger sequencing was performed by a dideoxynucleotide chain termination reaction using BigDye Terminator 1.1 (Applied Biosystems) and the above primers. Separation was performed by capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems). Sequences were analyzed by alignment to the Ensembl sequence in NCBI BLAST as well as by visual inspection in Sequence Scanner v 1.0 (Applied Biosystems).

Methylation specific PCR

Promoter methylation was studied by methylation-specific PCR [25], using primers specific for the unmethylated (U) and methylated (M) sequence. in the SDHAF2 and SDHD gene promoters using previously described methodology [26]. Bisulfate conversion of 400 ng of tumor DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). One µl of product was used in each PCR (U and M), using HotStar Taq Polymerase (Qiagen, Hilden, Germany) with the following conditions: 95°C for 15 min, 38 cycles of (94°C for 40s, 58°C for 40s, 72°C for 40s), 72°C for 5 min and hold at 4°C. PCR products were separated in 1.5% agarose gels containing ethidium bromide, and visualized with UV light. Methylated, unmethylated and non-converted wild-type DNA from the EpiTect PCR Control DNA Set (Qiagen) was used as controls. Bisulfite-converted blood-DNA samples from four patients were also included as controls.

The gene *HIC1* (hypermethylated in cancer 1), which has previously been shown to be hypermethylated in VHL-associated PCCs [27], was analyzed as a control, using methylation-specific PCR with previously published primers [28].

Quantitative mRNA expression analysis

Expressions of *SDHAF2* and *SDHD* mRNA were investigated using reverse transcription quantitative real time PCR (RT-qPCR) in those tumors for which RNA was available. RNA integrity was validated by microfluidic analysis on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). 500 ng of total RNA was transcribed into cDNA with Maxima First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA). Gene expression was measured on the 7900HT Fast Real-Time PCR System (Applied Biosystems), using pre-designed primer-probe mixes covering exon boundaries [26] and the TaqMan® Fast Universal PCR Master mix (Applied Biosystems). *GUSB* and *HPRT1* (Applied Biosystems) were used as reference genes and were analyzed simultaneously with *SDHAF2* and *SDHD* for all samples. These reference genes were selected since they have been shown stable expression in several tumor forms [29] and had expression levels comparable to *SDHAF2* and *SDHD* in our samples. cDNA transcribed from 20 ng of total RNA was used in each reaction, and all reactions were performed in triplicates. Normalized relative expressions of *SDHAF2* and *SDHD* were

calculated according to established methods [30] as follows: mean threshold cycle (C_T) value was calculated for each sample and gene by taking the mean of the PCR replicates. A normalized value (ΔC_T) was calculated by subtracting the mean of $C_{T(GUSB)}$ and $C_{T(HPRT1)}$ from $C_{T(SDHAF2)}$, and finally the relative expression was obtained as $2^{-\Delta C_T}$.

Immunohistochemistry

Immunohistochemistry was performed on 4–5 μ m thick formalin-fixed and paraffin-embedded sections from each tumor sample in an automated system (BondTM—maX, Menarini, Florence, Italy) according to the recommendations of the manufacturer. Anti-SDHB (mouse monoclonal, clone 21A11, Abcam, Cambridge, UK, dilution 1:1200, EDTA 30 min) was used as primary antibody. Sections were then lightly counterstained with hematoxylin. Appropriate positive and negative controls were analyzed in parallel. The relative staining intensity was graded -, +, ++ and +++ comparing adrenal medulla with adrenal cortex.

Results

Frequent LOH at SDHAF2 and SDHD

From 41 investigated VHL-associated PCCs, LOH or allelic imbalance indicative of LOH were identified in 22 (54%) and 21 (51%) tumors for *SDHAF2* (Fig. 1a+1b) and *SDHD*, respectively (Fig. 1c+1d). Concerning *SDHAF2*, another 3 tumors (7%) showed signs of LOH but this could not be fully determined (one or more allele ratios >0.7 but <0.8 and/or inconclusive at visual inspection). Regarding *SDHD*, this applied to 8 (20%) tumors. Normal heterozygosity with no signs of LOH (allele ratios between 0.9 and 1.0 and/or no signs of loss in electropherograms) for *SDHAF2* was found in 16 (39%) tumors. Concerning *SDHD*, 12 (29%) tumors showed normal heterozygosity.

Screening for SDHAF2A and SDHD mutations

No *SDHAF2* or *SDHD* mutations were identified by direct sequencing in 39 informative PCCs. A common polymorphism (rs17702, C→T) in the 3'UTR region of exon 4 was detected in heterozygous

form in 6 tumors (15%) and, where available, the corresponding blood samples displayed the same genotype as the tumors. In tumors with LOH, the peak size of one of the alleles was clearly reduced and sometimes hardly distinguishable. Allele frequencies of rs17702 did not differ significantly between the patients and 113 HapMap-CEU individuals reported at Ensembl ($p=0.12$, two-tailed Fisher's exact test).

Promoter methylation analyses

Methylation specific PCR for *SDHAF2* and *SDHD* investigated in 27 PCC samples showed no promoter methylation. All samples yielded PCR products when using primers specific for unmethylated DNA but not when using methylation-specific primers (Fig. 2). In order to study the methylation status of our samples, the gene *HIC1* was used as a control in methylation-specific PCR. *HIC1* was found to be methylated in all tumor samples (Fig. 2) but not in any of the tested blood samples.

Reduced SDHAF2 and SDHD expression

The mean relative *SDHAF2* mRNA expression ($2^{-\Delta CT}$), normalized against the reference genes *GUSB* and *HPRT1*, was 2.2 times lower in tumors with LOH/allelic imbalance compared to tumors with normal heterozygosity (0.34 ± 0.09 and 0.74 ± 0.31 , respectively). For *SDHD*, the mean relative expression was 2.9 times lower tumors with LOH/allelic imbalance than those without (0.29 ± 0.05 and 0.78 ± 0.24 , respectively). Differences in means were significant for both genes, $p=0.0052$ for *SDHAF2* and $p=0.042$ for *SDHD*, using two-tailed Student's t-test (Fig. 3).

Immunohistochemistry

SDHB protein expression intensity determined by immunohistochemistry was lower in the 6 analyzed PCCs (adrenal medulla) than in corresponding adrenal cortex (Fig. 4a + b). However, no obvious correlation was observed between the perceived staining intensity and LOH and/or levels of mRNA expression.

Discussion

In this study, we show that loss of *SDHAF2* and *SDHD* are frequent events in VHL-associated PCCs. More than 50% of our tumor samples clearly displayed LOH at the *SDHAF2* and *SDHD* regions. Another 7% and 20% of the PCCs showed signs of LOH/allelic imbalance at the *SDHAF2* and *SDHD* regions, respectively, but were inconclusive since the allele ratios were >0.7 but <0.8 . A possible explanation for this could be a heterogeneity of the tumors, which has recently been shown in PCCs [31]. For both *SDHAF2* and *SDHD*, LOH was associated with a significantly ($p<0.01$ and $p<0.05$, respectively) decreased levels of mRNA expression.

Somatic mutations of *SDHAF2* or *SDHD* were not detected in the investigated VHL-associated PCCs. Concerning *SDHAF2*, this is in agreement with earlier studies since *SDHAF2* germline mutations have so far only been found in patients with PGLs but not in PCCs [22, 32]. In contrast, mutations in *SDHD* [33], *SDHB* [34], *SDHC* [35], and *SDHA* [36] were initially found in PGLs but later also in PCCs (*SDHD* [37, 38], *SDHB* [34], *SDHC* [11], *SDHA* [39]).

Promoter methylation of *SDHAF2* or *SDHD* was not found in any of the investigated tumors. We did, however, detect promoter methylation of *HIC1* a gene that has previously been found to be hypermethylated in VHL-associated PCCs [27] in all our investigated tumors (but not in blood) and can thus conclude that the absence of promoter methylation is not due to complete absence of methylation in our samples. Presence of methylation at sites that we did not analyze, however, cannot be excluded.

If *SDHAF2* or *SDHD* would function as tumor suppressor genes in these tumors, one would expect a second mechanism of gene inactivation in addition to LOH in accordance with Knudson's two-hit model [40, 41]. Absence of mutations and promoter methylation may be in agreement with the putative maternal imprinting of *SDHAF2* and *SDHD* [42], but may also be suggestive of other or additional important target genes on chromosome 11q. Therefore, despite the lack of both somatic mutations and methylation in *SDHAF2* and *SDHD*, the significant down-regulation of both genes on the mRNA level in tumors with LOH suggested a pathogenic role of these genes in VHL-associated

PCCs. We therefore analyzed the expression of the protein SDHB in our PCCs as it is considered a universal marker for loss of any of the SDH subunits [23]. Actually, weak SDHB staining has been reported in VHL-associated PCCs [43]. Weak SDHB immunostaining of tumor tissue could be confirmed in the majority of the tumors investigated. However, we did not find any correlation between the mRNA levels of *SDHAF2* and *SDHD* and the expression of SDHB, making it less likely that either gene plays a major role in the development of these tumors even though the number of tumors investigated with IHC was very limited in this study.

In summary, we show a high frequency of allelic imbalance indicating LOH of both *SDHAF2* and *SDHD* in VHL-associated PCCs that was associated with a significantly decreased expression of both genes on the mRNA level. Intensity of SDHB IHC staining, however, correlated with neither LOH nor altered mRNA levels. This and the absence of both mutations and/or methylation indicates that the studied genes *SDHAF2* and *SDHD* are less likely to contribute a key genetic event for mitochondrial complex II change and subsequent formation of PCCs in carriers of *VHL* germline mutation. Other key genetic events on 11q remain to be looked for.

Declaration of interest

The authors declare that there is no conflict of interest that could prejudice the impartiality of this scientific work.

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TABLE 1. Details of microsatellite markers used for LOH analyses.					
Markers flanking the <i>SDHAF2</i> locus					
Marker Name	Position Ensembl [Mb]	Heterozygosity	Forward and reverse primer sequences [5' to 3']	Annealing temp.	Product size [bp]
D11S4191	60.00	0.87	F: 6-FAM-GCAAGATGGCCAATTAGAAG R: CCTTAGTTATTTTGGTTGGAATGT	55 °C	118-142
D11S1765	60.78	0.79	F: 6-FAM-CAGAAATGCCACCCAGAGAG R: TTCCGGAGTTTGCACAATCT	57 °C	234-252
D11S4076	61.36	0.77	F: 6-FAM-CATGAATGCTCTTGTCCC R: AACCCCCTGGAAAATAGACT	55 °C	151-163
D11S1883	63.37	0.73	F: 6-FAM-GTGGCTCACGCCTGTAATC R: TCTTTCTGTTGGTTTCGGATCT	59 °C	177-219
Markers flanking the <i>SDHD</i> locus					
D11S897	105.74	0.66	F: 6-FAM-ATATCACAGAGGAGTCCCCT R: AACTTGAAGAGCAGAAAGGG	55 °C	98 - 118
D11S1793	109.86	0.68	F: 6-FAM-AGTCATGCATCCTCCCTGTA R: ATCCTGAACACATTCCTCAA	56 °C	100 - 140
D11S1986	111.3	0.69	F: 6-FAM-CCAAGATCGGACCATTGTAC R: AAGCCTAAAGGTGATGGGTC	57 °C	188 - 248
D11S3179	112.7	0.74	F: 6-FAM-CTTCCTACCAAAGGGGC R: ATCAATCCATCAGTGGGG	55 °C	179 - 197
Positions are according to Ensembl (http://www.ensembl.org/index.html). Heterozygosity measures were obtained from Marshfield (http://research.marshfieldclinic.org/genetics/GeneticResearch/compMaps.asp). Primer sequences were obtained from NCBI. F = forward primer, R = reverse primer.					

TABLE 2. Primers and conditions used for mutation screening			
The <i>SDHAF2</i> gene			
Exon no.	Forward and reverse primer sequences [5' to 3']	Annealing temp.	Product size [bp]
1	F: CTTCCGGCTCAGCTCCTC R: TATCGGGCAGACGAACTCTC	57 °C	118-142
2	F: AAAGATGTTGTGGTTTGTTC R: CAGGAAGCTGCCCCGATTTA	55 °C	234-252
3	F: CCCAGGAGTAGGAGTCTTGGT R: TCAGCCTAAACTGTCCTATTTTCA	56 °C	151-163
4	F: CCTGAGCATTGACTGACTATGG R: GGGGGAAAGTTTTCTATAGTGCT	56 °C	177-219
The <i>SDHD</i> gene			
1	F: GTGCAGTAAACTGCGCCTTC R: CCTTCGGGTAAACATCTGGA	58 °C	98 - 118
2	F: CTTACAGTAACCCCAGTGAAA R: TTTGACAAAGTTGGACACAAGAG	56 °C	100 - 140
3	F: GCATTGAGATACCCTTGTGCT R: TCAACAAATTTAGGGCATTTCA	56 °C	188 - 248
4	F: GGAGTGGCAAATGGAGACAT R: TCCTCTCAAACCTTTTCCTCA	58 °C	179 - 197
Primer sequences were obtained from NCBI. F = forward primer, R = reverse primer.			

Figure legends

Fig. 1

LOH analysis of microsatellite loci flanking the *SDHAF2* and *SDHD* gene loci in paired blood and PCC tumor samples from VHL patients. Representative examples are shown where the tumor exhibits **a) + c)** LOH (indicated by arrows), present in 51-54% of the samples, and **b) + d)** normal heterozygosity. Allele ratios were calculated as previously described [24]. The ratio between tumor and normal allele ratios, i.e. $T1:T2/N1:N2$ where T1 and N1 are the peak heights of the shorter allele in the tumor and normal sample, respectively, and T2 and N2 are the peak heights of the longer allele. In cases where the ratio was > 1 , it was converted using $1/(T1:T2/N1:N2)$.

Fig. 2

Methylation-specific PCR in eight representative VHL-associated PCCs. Wild-type non-converted DNA (wt), unmethylated bisulfite-converted DNA (U) and methylated bisulfate-converted DNA (M) were used as controls. For the *SDHAF2* and *SDHD* promoters, only the unmethylated sequence was amplified in the samples, thus none of the tumors showed any signs of methylation. For *SDHAF2*, the methylated control showed a weak band with non-methylation-specific primers, possibly because of unspecificity of primers or a small amount of unmethylated DNA in this control, however this does not interfere with the interpretation of the results. Methylation-specific PCR of the gene *HIC1* that served as a control showed methylation in all samples.

Fig. 3

Mean relative expression of *SDHAF2* and *SDHD* mRNAs in VHL-associated PCCs. Separate bars are shown for *SDHAF2* in cases with LOH (n=8) or normal heterozygosity (n=4) as well as for *SDHD* in cases with LOH (n=7) or normal heterozygosity (n=6). The expression was normalized against the reference genes *GUSB* and *HPRT1*. The mean expression was significantly lower in tumors with LOH

at *SDHAF2* ($p=0.0052$) or *SDHD* ($p=0.042$) compared to tumors with normal heterozygosity. Error bars show standard deviations.

Fig. 4

Immunohistochemical detection of SDHB expression in two representative PCCs (**a+b**). Both tumors (adrenal medulla; arrow) showed less intense expression than the corresponding adrenal cortex (*).

Figure 1

Figure 2

Figure 3

SDHAF2

SDHD

Figure 4